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Multi-gene co-expression can improve comprehensive resistance to multiple abiotic stresses in *Brassica napus* L.

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ABSTRACT

Rapeseed (*Brassica napus* L.) is an important oil crop worldwide. For current *B. napus* production, it is urgent to develop new varieties with higher seed productivity and increased stress tolerance for better adaptation to the abiotic stresses as a result of global climate change. Genetic engineering, to some extent, can overcome the limitations of genetic exchange in conventional breeding. Consequently, it considered as an effective method for improving modern crop breeding for *B. napus*. Since crop stress resistance is a polygenic complex trait, only by multi-gene synergistic effects can effectively achieve the comprehensive stress resistance of crops. Hence, in the present study, five stress resistance genes, *NCED3, ABAR, CBF3, LOS5*, and *ICE1* were transferred into *B. napus*. Compared with wildtype (WT) plants, the multi-gene transformants K15 exhibited pronounced growth advantage under both normal growth and stress conditions. Additionally, K15 plants also showed significantly higher resistance response to multiple stresses at seed germination and seedling stages than WT plants. Furthermore, K15 plants had significantly higher leaf temperature and significantly lower stomatal aperture and water loss rate than WT plants, which indicated that the water-holding capacity of K15 plants was significantly superior to that of WT plants after stress treatment. In addition, K15 plants had significantly higher abscisic acid (ABA) content and significantly lower malondialdehyde (MDA) content than WT plants. In conclusion, the above results suggested that multi-gene co-expression could rapidly trigger plant stress resistance, reduce the stress injury on plants and synergistically improve the comprehensive resistance of *B. napus*.

1. Introduction

Rapeseed is one of the major oil crops in the world and mainly includes *Brassica napus*, *Brassica juncea* and *Brassica rapa*. Among these, *B. napus* is the main rapeseed cultivar and cultivated worldwide [1]. Due to global climate change and the frequent occurrence of extreme weather, drought, extreme temperature and other natural disasters have seriously restricted *B. napus* production [2]. Therefore, the necessity of develop novel *B. napus* varieties especially with high yield, high quality, and robust stress resistance has become of paramount importance. Regarding the breeding principle, *B. napus* breeding technology could be divided into four categories: hybrid-breeding, cell engineering breeding, DNA molecular marker-assisted breeding and genetic engineering breeding.

Conventional breeding in *B. napus* mainly takes advantage of heterosis to achieve complementary effects among various lines through hybridization. However, this hybrid-breeding method is not only laborious and it takes many years [3]. The first establishment of cell-engineering-based breeding technologies, including haploid breeding, somatic hybridization and tissue culture for asexual reproduction, back to the 1970s, which have also been applied in *B. napus* breeding. Although cell-engineering technology has overcome the limitations of incompatibility in distant hybridization, both cell engineering breeding and hybrid breeding just only achieve cell fusion and genetic exchange for the same species or close relatives but not for a wide genetic exchange across different phyla or genera. Furthermore, unfavorable genes that are closely linked to the target trait influence both methods, and such susceptibility becomes the primary factor that limits the application of hybrid breeding and cell engineering breeding. Moreover, DNA molecular marker-assisted breeding has many

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advantages, such as rich information to be marked, a great number of co-dominant molecular markers and marker detection unaffected by growth/development stages and tissues/organs [4]. However, for this method, it is the DNA marker linked to the target trait but not the target gene that is to be directly manipulated.

The advent of transgenic technology has opened new avenues in the improvement of crop plants. It offers the possibility of genetic exchange between different species and even across different phyla and genera. Moreover, this technology manipulates the target gene that directly controls a trait. Recently, with the emerging improvements of genetic engineering technology, introducing exogenous genes into target crops for quality and resistance improvement through transgenic technology has been widely used [5]. According to incomplete statistics, China has nearly 300 patent applications of transgenic *B. napus* as of 2013, which involves nearly 60 genes [6]. By 2015, China had published a total of 497 papers in transgenic *B. napus,* with nearly 7,000 citations [7]. Target genes in *B. napus* transformation mainly include two categories: quality improvement genes and stress resistance genes. To the best of our knowledge, for most *B. napus* transformants, only single target genes were transformed. Stress resistance of most plants is a polygenically controlled quantitative trait [8]. Although it is a certain improvement effect on crop resistance, single gene transformation cannot always effectively enhance crop resistance. Hence, in recent years, these deficiencies prompted researchers to attempt using multi-gene transformation to improve crop resistance [9]. In 2003, Zhao's established several researches, first transferred multiple genes into plants separately and then used plant crossing to successfully obtain multi-gene transformants. In the same year, expression vectors for the multiple genes were constructed, and all the genes were then transformed with re-transformation to obtain multi-gene transformants [10]. Whereas, these methods are time-consuming with a long cycle and are laborious, this greatly limits their applications. As early as 1998, there were researches on using the co-transformation method to obtain multi-gene transformants, but co-transformation efficiency was related to plasmid ratios and transformants might not acquire all transgenes [11,12]. Constructing multi-gene expression vector and transferring into plant based on one genetic transformation event seems to be a valid approach to obtain multi-gene transformants, but its prerequisite is to construct the multi-gene transformation vector. Urwin et al. have proposed using internal ribosome entry sites ($IRES_S$) from various viruses as the spacer sequence of a polycistron, which, upon transcription, can recruit ribosomes through these spacers to achieve translation of single cistrons [13]. Since eukaryotic genes exist in the form of single cistrons, polycistrons composed of multiple genes do not necessarily render the real form of transcription in plants and could also result in a low accuracy of gene expression. Alternatively, a multi-gene expression vector can be obtained by construction of single gene expression cassettes followed by the assembly of these single gene expression cassettes [14]. The assembly method has more advantages over the previous method of Urwin et al. Taking advantage of Gateway technology and the multiple-round *in vivo* site-specific assembly (MISSA) method, five stress responding genes were successfully constructed into a multi-gene transformation vector pABA-oriT and the vector was transformed into *Agrobacterium* strain COR308 [15]. In this study, we transformed this multi-gene expression vector into *B. napus* in order to improve its comprehensive resistance to various abiotic stresses.

2. Materials and methods

2.1. Plant materials, strains and primers

The recipient material for genetic transformation was a restorer of the cytoplasmic male sterile (CMS) line in *B. napus*, 09Y42, developed by the State Key Laboratory of Cotton Biology, Henan University. The *Agrobacterium* strain used for genetic transformation was COR308. This strain carries the binary vector pABA-oriT, in which 5 stress responding genes *NCED3* (Nine-Cis-Epoxycarotenoid Dioxygenase 3), *ABAR* (ABA Receptor, magnesium-chelatase subunit chlH), *CBF3* (C-repeat Binding Factor 3), *LOS5* (molybdenum cofactor sulfurase, ABA3), and *ICE1* (interactor of little elongation complex ELL subunit 1) have been cloned by the MISSA (multiple-round *in vivo* site-specific assembly) method [15]. All primers used in current investigation were designed using Primer Premier Software (version 5.0) (Premier Company, Canada) and were synthesized by Sangon Biotechnology Limited Company, Shanghai, China (see the Supplementary Information, Table S1).

2.2. Transformation of Brassica napus and identification of homozygous lines

B. napus were transformed by floral dip method [16,17] and moderately revised. The *B. napus* recipient materials to be transformed, 09Y42, were transplanted in the greenhouse after low-temperature vernalization in the field. Then, plants were growing into the early flowering stage in a controlled greenhouse at 25–28 °C, 35–40% relative air humidity and light extended for 2 more hours each morning and evening for managing the photoperiod. To prepare the *Agrobacterium* transformation solution, *Agrobacterium* was inoculated into the yeast extract peptone (YEP) liquid medium and oscillated (220 rpm) at 28 °C until the OD_{600} value reached 1.2-1.5. The culture was centrifuged at 4,000 rpm for 15 min under room temperature condition. The resulted pellet was suspended in half-strength MS liquid medium and diluted to a final OD_{600} of 0.8-1.0. Then, 0.05% Silwet-L77 was added immediately preceding the transformation. Followed by immersed inflorescences into the transformation solution for 90 s and were subsequently bagged to maintain adequate moisture. The transformation was repeated every other day, and three transformations were performed overall. Under greenhouse conditions, the harvested seeds were sown to screen transformants. After 7 days, when the first true leaf was emerging, plants were sprayed with 50 mg/l Basta once per day for three days. For a second screening, 50 mg/l hygromycin was further applied to Basta-resistant plants at the fully stretched third true leaves during the four-leaf stage. For plants, that conferred double resistance of Basta and hygromycin, DNA was extracted from leaves and a polymerase chain reaction (PCR) was performed followed by sequencing for identification of the insertion. The positive transformants were self-pollinated for multiple generations to obtain homozygous lines.

2.3. Seed germination and seedling growth index determination

The seeds with full-grain and uniform size were selected for seedling growth index determination. Seeds were surface-sterilized and sown by spot sowing on basal MS medium (CK) or containing 100 mM NaCl or 200 mM mannitol and or 10 μM abscisic acid (ABA) and cultures were kept in dark for two days. Then, all cultured were maintained at 25 °C with 16/8 h of light/dark (72 $\mu mol/m^2/s$ of light intensity), and relative humidity of 35–40%. The germination rate was determined on days 3, 4, 5 and 9 after seed sowing. On day 10, root length, plant height, fresh weight and dry weight were measured, and the seedling length vigor index (SLVI) was calculated as (average root length + average plant height) × highest germination rate [18]. Each experiment was repeated thrice at different times.

2.4. Gene expression analysis

For stress treatment, seedlings grown for 7 days on half-strength Hoagland nutrient solution were subjected to 1/2 Hoagland drought stress (15% PEG_{6000}) or salt stress (150 mM NaCl). To determining the expression levels of the target gene and the *GUS* gene [15], RNA was extracted from whole plants at 0 h, 6 h, 12 h and 24 h after beginning of the stress treatments.for RT-PCR. Each experiment was performed three times. The qRT-PCR was conducted using GoTaq qPCR Master Mix (Promega Biotechnology) on an ABI 7500 FAST real-time PCR machine

(Applied Biosystems, USA) with a final volume of 10 μL per reaction. Each reaction mixture contained 5 μL GoTaq Mix (GoTaq qPCR Master Mix 2X), 2.0 μL cDNA template, 0.5 μL each primer (1.0 μM), and 2 μL nuclease-free water. Each reaction was performed in triplicate. The cycling parameters were 95°C for 5 min, followed by 40 cycles at 95°C for 15 s and 60°C for 30 s. The *B. napus* Actin1 gene was used as the internal reference gene. The relative gene expression levels were determined using the $2-\Delta Ct$ method70.

Seedlings with 7 days old on 1/2 Hoagland liquid culture (CK) were treated with various stress conditions, including 15% PEG-6000, 150 mM NaCl, cold (4 °C), heat (40 °C) and ABA (30 μmol/L) for 12 h. RNA was extracted from whole plants for RNA-Seq analysis. Corresponding to the five stress treatments, K15 samples were named A, B, D, E, F and G, whereas WT samples were named as a, b, d, e, f and g. Total RNA was extracted with TRIzol according to the manufacturer's instructions (Invitrogen, USA).

Primary sequencing data were produced using Illumina HiSeq™ 2000 and have been deposited in the NCBI (SRA, http://www.ncbi.nlm.nih.gov/Traces/sra) with accession number SRP109808. The RPKM method was used to calculate gene expression levels and screening on different expression genes. To reveal the internal relationships among the selected DEGs and their biological processes and pathways, we constructed networks and performed network analysis based on regulation networks in Arabidopsis. The cluster analysis is performed by Cluster 3.0 and JavaTreeview, where the average linkage method is used during the clustering processes.

2.5. Stress resistance phenotype and physiological index analysis

Plant culture and stress treatment: *B. napus* seeds were sown on pots containing vermiculite, nutrient soil (3:1, volumetric ratio) and incubated at 25 °C under a 16-h photoperiod at light intensity of 72 μmol/m^2/s and 35–40% relative humidity. The high-temperature stress treatment was applied from sowing date to the bolting stage by setting the temperature to 35 °C. While, at the four-leaf stage seedlings were subjected to low-temperature stress (0 °C) for 12 h. Drought stress treatments were also carried out at the four-leaf stage. To simulate drought stress, the surface of the nutrition pots were immersed in 20% PEG-6000 solution for 12 h.

Leaf temperature measurement: Four-leaf-stage seedlings at 35% and 5% of soil water content were used for leaf temperature measurement. The second and third true leaves at the same position and size for both K15 and WT genotypes were selected for measurement. Leaf temperature was measured *in vitro* by far-infrared thermography (SC-3000, Thermo USA). For each treatment, five plants were measured each time, and the experiment was repeated three times.

Leaf water loss measurement: The second true leaves of the four-leaf-stage seedlings were selected for the determination of leaf water loss. The measurement time lasted 12 h, from 10:00 to 22:00. Real-time data were recorded using the Sartorius moisture analyzer (QUINTIX-224-1CN, Germany). The data was recorded for five plants each time, and the experiment was conducted three times.

Stomatal aperture measurement: The second true leaves of the four-leaf-stage seedlings cultured in the nutrition pots were selected for the determination of stomatal aperture. Taking the middle vein of the leaf as the central axis, the leaf was divided into two halves. For each half of the leaf, the lower epidermis was placed upward in the buffer (60 mM KCl, 10 mM 2-(N-morpholino) ethanesulfonic acid) for 5 h at 285 μmol/m^2/s of light intensity to induce stomatal aperture. Upon tearing the lower epidermis and removing the mesophyll cells, the stomatal aperture was measured. The first half of the leaf was measured at 8:00 in the morning, whereas the other half of the same leaf was allowed to grow until measured at 10:00. The stomatal aperture can be presented as a percentage (%) of the ratio of stomatal width to stomatal length. Three plants were measured each time, with the number of stomata approximately 230-270. The experiment was repeated three times.

ABA and MDA content determination: The ABA and MDA content were determined following the method described by Zhai et al [19].

2.6. Network construction and analysis

Based on the NCBI database (https://www.ncbi.nlm.nih.gov), we downloaded the gene regulatory information for the five *A. thaliana* genes AT5G13630, AT3G14440, AT1G16540, AT3G26744 and AT4G25480. The corresponding gene network was drawn by Gephi [20]. *B. napus* homologous genes for the genes in the constructed network were predicted by means of synteny-based methods in the CoGe database (https://genomevolution.org) [21]. Orthologous genes of *B. napus* were predicted in the target genomes of *A. thaliana* through the identification of a conserved syntenic region *via* SynFind with the following parameters: last comparison algorithm, minimum number of four genes, and unlimited syntenic depth. Putative paralogous genes in *B. napus* were identified *via* SynMap by the following parameters: last algorithm, 20 genes for the maximum distance between two genes, and five genes for the minimum number of aligned pairs. The coverage depth of *B. napus versus* A. thaliana was determined at 6:1.

3. Results

3.1. Multi-Gene transformants significantly increased expression levels of target genes and exhibit significantly enhanced tolerance to PEG6000 and NaCl stress in the germination stage

In order to enhance resistance to abiotic stress, such as drought, salinity, and low temperature, five genes involved in both the ABA-dependent and ABA-independent pathways of plant response to abiotic stresses were assembled into the T-DNA of the pABA-oriT vector [15]. The genes involved in the ABA-dependent pathway are *NCED3*, *LOS5*, and *ABAR*, while the genes involved in the ABA-independent pathway are *ICE1* and *CBF3* [22–26]. Three marker genes (*HYG*, *BAR*, and *GUS*) were also assembled into the T-DNA of pABA-oriT for the selection and identification of transformants (Supplemental Figure S1).

The *Agrobacterium* strain COR308, which contains the transformation vector pABA-oriT, was transferred into *B. napus* by the flower-dip method at the early flowering stage. Approximately, 4,100 T_1 seeds were harvested and then sown in the greenhouse. Seven-day-old plants, when the first true leaves emerged, were sprayed with 50 mg/l Basta. The transformants grew normally by conferring Basta resistance, but non-transformants gradually turned to yellow and finally perished. Following Basta selection, the Basta-resistant seedlings were grown to their four-leaf stage for the second stage selection against 50 mg/l hygromycin at the fully expanded third true leaves. After 5 days, leaves of the non-transgenic plants had densely distributed brown spots in the hygromycin application region, and gradually loss chlorophyll and turned dry. However, transformants grew normally by conferring hygromycin resistance (Fig. 1A). For PCR identification of plants that conferred double resistance to Basta and hygromycin, DNA was extracted from young leaves (Fig. 1**B and C**). Transformants that positively identified *via* PCR amplification products sequence were transplanted in the field to grow and complete their life cycle. Thereafter, 9 primary transformants were obtained in the T_2 generation, two of them were used for further experiment. Transformants were self-pollinated to produce homozygous transformant lines in T_4 generation, which named K15. The corresponding non-transformant line was referred as WT. The GUS activity assay showed that the *GUS* gene expressed in the K15 plants (Fig. 1**D and E**).

The structures of the expression cassettes revealed significant difference among the five target genes. The promoter of *ICE1* and *LOS5* was *pSuper*, whereas the promoter of *CBF3*, *ABAR* and *NCED3* was *pRD29A*, which is stress-inducible, because constitutive overexpression of *CBF3*, *ABAR* or *NCED3* in transgenic Arabidopsis would cause

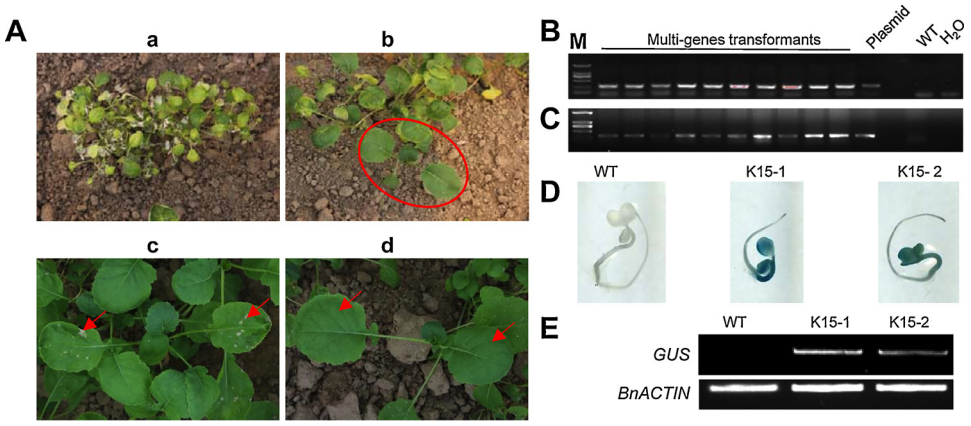

Fig. 1. Identification of multi-gene transformants and GUS activity assay. A: PPT and Hyg resistance screening; a: PPT-negative plants; b: PPT-positive plants; c: Hyg-negative plants; d: Hyg-positive plants. B: PCR amplification for the BAR gene. C: PCR amplification for the HYG gene. In B and C, M represents Marker DL 2000. D: GUS activity assay. E: RT-PCR for the *GUS* gene expression.

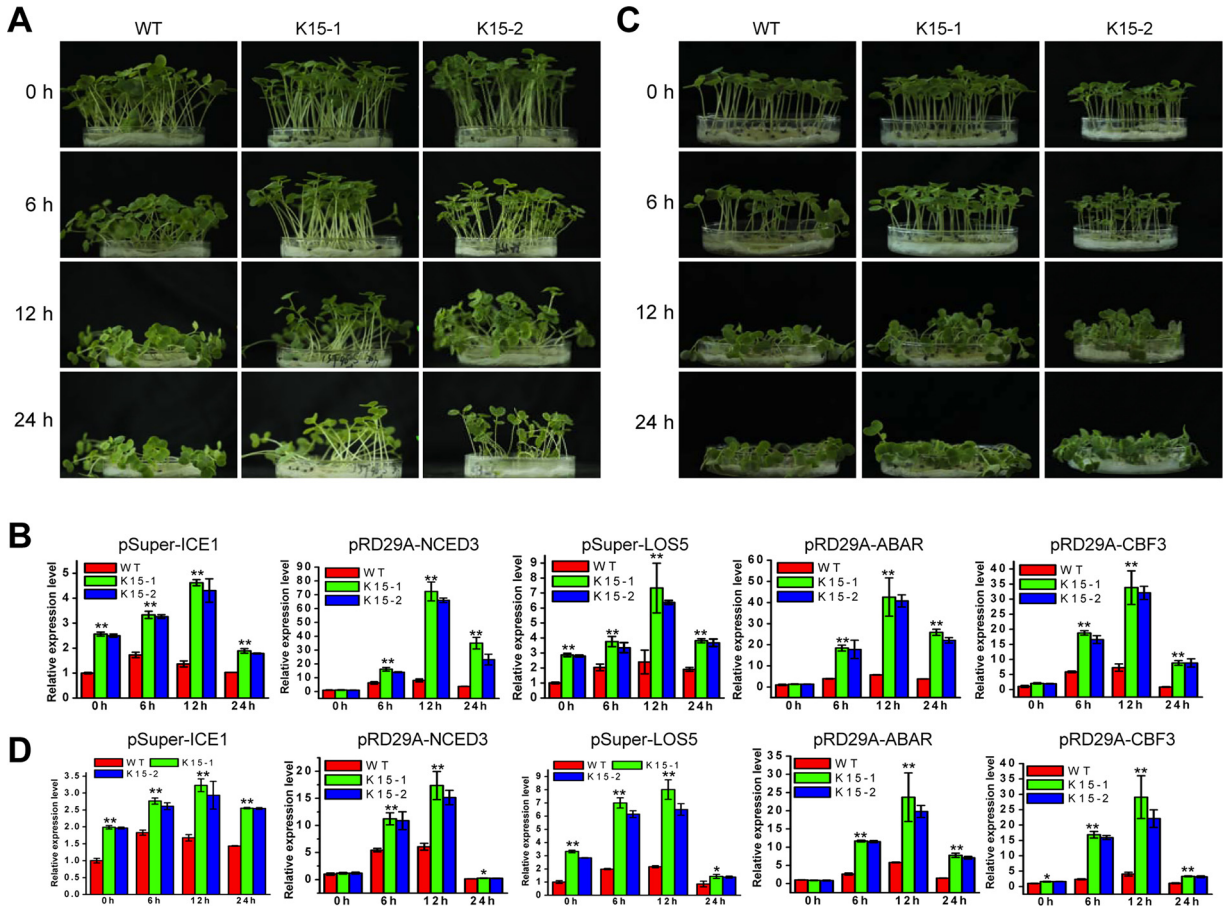

Fig. 2. Seedling phenotype and gene expression analysis of multi-gene transformants under PEG6000 and NaCl stress treatment. A and C show the seedling phenotypes under PEG6000 and NaCl stress, respectively. B and D are corresponding qRT-PCR analyses for the target gene under PEG6000 and NaCl stress, respectively. **: $P \leq 0.01$; *: $P \leq 0.05$.

abnormal growth and development phenotype [24,25]. As seen from Fig. 2A, K15 seedlings exhibited significantly higher tolerance upon treatment with 15% PEG6000 as compared with WT seedlings. After PEG6000 treatment, WT seedlings showed a serious degree of plant lodging, which failed to stand upright at 6 h, wilted at 12 h, and were completely lodged and turned dry at 24 h. In contrast, K15 seedlings could stand upright at 6 h after PEG6000 treatment, started to wilt at 12 h, and then started to have some degree of plant lodging at 24 h (Fig. 2A). Moreover, qRT-PCR analysis showed that the five target genes had different expression patterns in K15 plants (Fig. 2B). Before PEG6000 treatment, the expression levels of *ICE1* and *LOS5* driven by the *pSuper* promoter in K15 plants were significantly higher than (approximately 3 times) those in WT plants. Upon PEG6000 treatment, the expression levels of *ICE1* and *LOS5* were up-regulated in both materials, but both genes showed a great increase in K15 plants than WT. Additionally, at 12 h, the expression levels of *ICE1* and *LOS5* in K15 and the expression level of *LOS5* in WT reached their peaks of expression abundance. An exception was that the peak expression level of *ICE1* in WT plants occurred slightly earlier than 12 h after treatment. In addition, similar results were obtained for the expression patterns of *CBF3*, *ABAR* and *NCED3* driven by the inducible *pRD29A* promoter. In the normal growth condition, nearly no differences in the expression abundance of the three genes between the WT and K15 plants were observed. On the other hand, after applied the PEG6000 treatment, the

expression abundances of the three genes increased sharply, and their expression levels reached the highest point at approximately 12 h after treatment in K15.The expression levels of *NCED3*, *ABAR* and *CBF3* reached up to 75, 45 and 35 times respectively at 12 h after PEG6000 treatment. In contrast, the expression abundance of the three genes increased only marginally in WT at this time (Fig. 2**B**).

After 150 mM NaCl treatment, the phenotype and gene expression pattern of K15 and WT seedlings showed similarities to those of K15 and WT seedlings after 15% PEG6000 treatment, yet some differences still existed (Fig. 2C). First, K15 seedlings treated by NaCl exhibited some extent stress resistance than WT seedlings; however, both K15 and WT seedlings treated by NaCl showed a more severe wilting pattern, and they turned dry and died at 12–24 h after treatment (Fig. 2**A and C**). Second, after NaCl treatment for 12 h, the expression levels of *NCED3*, *ABAR* and *CBF3* were up to 20, 25 and 30 times, respectively in K15 seedlings, and were obviously lower than drought treatment (Fig. 2D). Third, compared with treatment by PEG6000, K15 seedlings treated by NaCl for 12 h demonstrated lower upregulated extent of the five target genes.

For instance, the expression levels of the three inducible genes, *NCED3*, *ABAR* and *CBF3*, in K15 plants after NaCl treatment for 24 h were 0.4, 8 and 3.5 times, respectively, but their expression levels were 35, 28 and 10 times respectively when K15 plants were treated by PEG6000 for the same hours (Fig. 2**B and D**).

3.2. The multi-gene co-expression enhanced heat and cold stress tolerance and brought down the low-temperature requirement during vernalization in Brassica napus

At the seedling and vegetative growth stages, K15 and WT plants were subjected to continuous high-temperature stress treatment at 35 °C. The results showed that the ability of K15 plants was higher for heat stress tolerance than WT plants. First, K15 plants had an absolute advantage in growth at the seedling and vegetative growth stages. More importantly, the main difference between these two was that K15 plants treated by high-temperature stress, even without undergoing vernalization by low temperature, could successfully pass the transition from vegetative growth to reproductive growth and further complete the life cycle. In contrast, WT plants which did not undergo vernalization would fail to enter the reproductive growth stage (Fig. 3A).

Notably, when K15 and WT plants grown in the greenhouse and not subjected to low temperature for vernalization, instead, both subjected to continuous drought treatment for two months, K15 and WT plants also exhibited phenotypic differences similar to those that were treated by high-temperature stress (Fig. 3B). On the other hand, when seedlings cultured in the nutrition pots and placed in growth incubation chamber (16/8 h light/dark, 25 °C, relative humidity 35–40%), and then subjected to low-temperature stress treatment at 0 °C for 12 h during the four-leaf stage, K15 plants were strongly surpassed WT plants under cold stress (Fig. 3C).

3.3. The multi-gene co-expression significantly enhanced growth potential and stress resistance

The tolerance to stress treatment during the germination stage was compared under three stress conditions (100 mM NaCl, 200 mM mannitol and 5 μM ABA). Recorded data for the seed germination rates from day 3 to day 9 after seed sowing under various stress treatments were significantly difference. In general, K15 seeds showed significantly higher germination rates than WT (Table 1). K15 seeds reached the highest germination rates of 100%, 99% and 98% on day 5 after treatment by 100 mM NaCl, on day 9 after treatment by 200 mM mannitol and 5 μM ABA, respectively. In contrast, the germination rates of WT seeds were lower under the condition of NaCl and mannitol treatment, which were 81% and 77%, respectively on day 9. Under ABA treatment, WT seeds reached the highest germination rate (97%) by day 9. Generally, under the three treatments, the germination of K15 was significantly faster than that of WT, especially under NaCl and mannitol treatment.

Furthermore, the root length and plant height were measured on day 10 after seed sowing. The results mentioned that the root length of K15 seedlings was significantly longer than that of WT under all three treatments. Moreover, the plant height of K15 seedlings was slightly higher (no significant difference) than that of WT seedlings under 100 mM NaCl and 200 mM mannitol treatment. However, it was significantly higher than that of WT seedlings under 5 μM ABA treatment (Table 1).

SLVI is an indicator of overall vigor during the seed germination stage and post-germination growth stages. The results revealed that the SLVI of K15 seedlings was significantly higher than that of WT seedlings under all three treatments (Fig. 4), which indicated that K15 plants exhibited significantly higher resistance to different stress treatments than WT plants during the seed germination stage and post-germination growth stages.

The ratio of the fresh weight of stressed seedlings to the fresh weight of non-stressed seedlings was defined as the relative fresh weight (RFW). Likewise, the relative dry weight (RDW) was defined except that the dry weight of seedlings was used. The obtained results showed that both RFW and RDW of K15 seedlings were higher than those of WT seedlings under all three-stress treatments. In more details, RFW of K15 seedlings was higher than that of WT seedlings ($P = 0.027$) under 200 mM mannitol treatment, but no statistically significant difference (Table 2). Therefore, data illustrated in Table 2 revealed that the declining extent of RFW and RDW for K15 seedlings were lower than those for WT seedlings after stress treatment. This indicated that under the same stress condition, K15 was less affected by stress, while its water-holding capacity and biomass accumulation were higher than those of WT.

3.4. The multi-gene co-expression significantly increase physiological and biochemical indexes in stress resistance and significantly decrease MDA content

Leaf temperatures were measured with a far-infrared thermography SC-3000 at the surfaces of the *in vitro* second and third true leaves at the four-leaf stage. The results showed that the leaf temperature of the second and third true leaves between the K15 and WT seedlings was not significantly different when the soil water content was approximately 35% (Fig. 5**A and B**). However, when the soil water content was reduced to approximately 5%, the *in vitro* leaf temperature of K15 seedlings was significantly higher than that of WT seedlings, with an average 2 °C and 1.5 °C higher for the second and third true leaves, respectively.

The result of the water loss rate (WLR) measurements at the second true leaf *in vitro* for K15 and WT showed that K15 leaf had a stronger water-holding capacity. The WLRs in K15 and WT leaves began to diverge significantly after 30 min. After 350 min, the WLR of K15 was only approximately 20%, whereas WLR of WT was 50% or higher. After 700 min, the WLR of K15 was close to 35%, while the WLR of WT was approximately 80% (Fig. 5E).

Stomata are the main channels for gas exchange and transpiration in plants. Further measurements of stomatal density and stomatal aperture for K15 and WT leaves found that there were no significant differences in stomatal density between the two, although the stomatal aperture of WT leaves was significantly greater than that of K15 leaves (Fig. 5**C and D**). Upon a light intensity treatment, the stomatal aperture of WT leaves increased extremely (P value by t-test was 1.34×10^{-8}). Although the stomata in K15 leaves were also light inducible, the stomatal aperture was only slightly increased compared with that of WT leaves (P value by t-test was 4.8×10^{-4}) (Fig. 5**C and D**).

Out of the expression cassettes of the five target genes, two were ABA synthesis-related genes (*NCED3, LOS5*), and one was an ABA signal

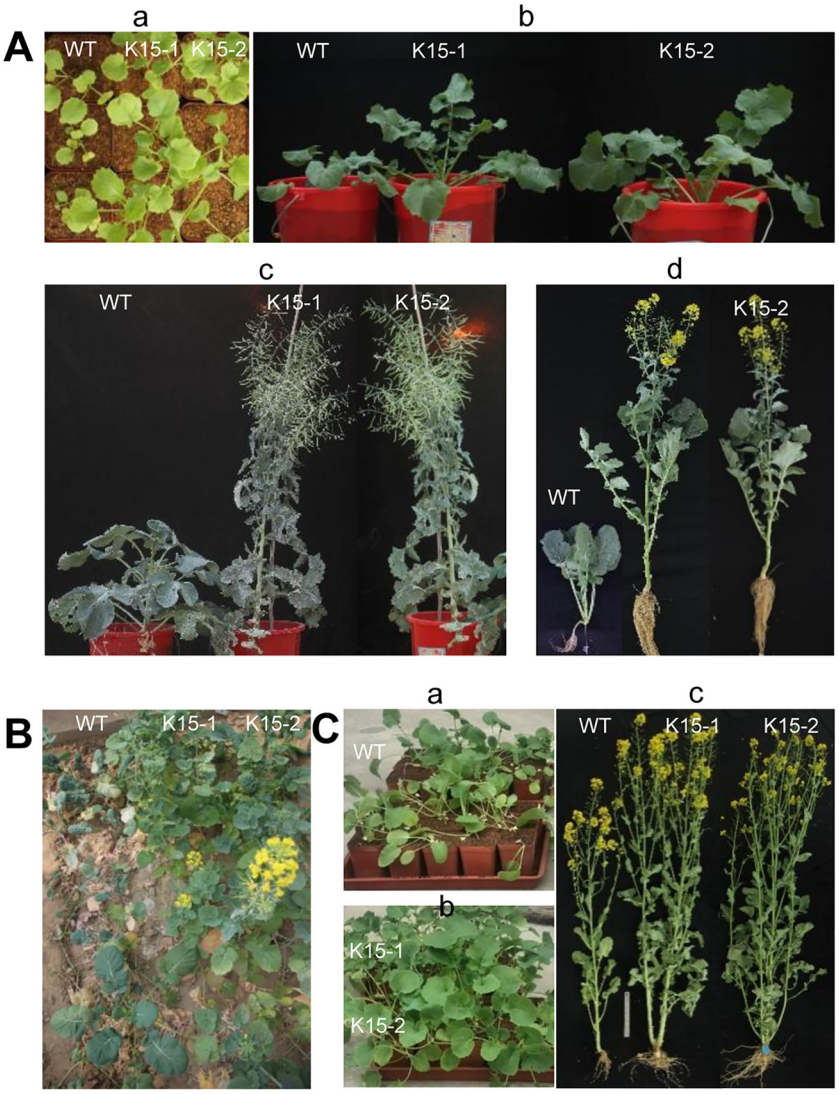

Fig. 3. Growth phenotype of multi-gene transformants under different stress conditions. A: High-temperature stress treatment. a: Seedling stage; b: Vegetative growth stage; c: Flowering stage; d: Reproductive growth stage. B: Phenotype during flowering stage after drought stress treatment; C: Phenotype after cold treatment. A: Seedling stage; b: Flowering stage.

pathway-related gene (*ABAR*). To verify the overexpression of these target genes, the ABA content in the K15 and WT plants was further measured. The results showed that, with no stress treatment, the contents of ABA were 357.8 ng/g and 299.3 ng/g in K15 and WT at 7–day-old seedlings, respectively (Fig. 6A), and 168.3 ng/g and 130.5 ng/g in the third true leaves of K15 and WT at four leaves stage, respectively (Fig. 6B). The content of ABA in K15 seedlings and leaves were significantly higher than in WT. On the other hand, with stress treatment, the contents of ABA were 507.7 ng/g and 380.6 ng/g in K15 and WT seedlings, respectively (Fig. 6A), and 267.9 ng/g and 190.9 ng/g in K15 and WT leaves, respectively (Fig. 6B). Upon stress treatment, the contents of ABA in K15 seedlings and leaves increased rapidly and had very significant differences compared with WT.

MDA reflects the degree of membrane lipid peroxidation in plant cells. Generally, plants under the stress conditions, such as drought, high temperature, saline and strong light, show membrane lipid peroxidation. The results showed that the MDA content in WT and K15 leaves with no stress treatments were 5.73 μM/g and 3.20 μM/g, respectively. However, the MDA content in WT and K15 leaves had rapid increases and reached 11.16 μM/g and 7.37 μM/g, respectively with

Table 1
Statistics of seed germination rates after different treatments.

Time (day)	Mock			100 mM NaCl			200 mM mannitol			5 μM ABA		
	WT	K15-1	K15-2	WT	K15-1	K15-2	WT	K15-1	K15-2	WT	K15-1	K15-2
9	100%	100%	100%	81%	100%	100%	77%	99%	98%	97%	98%	99%
5	100%	100%	100%	75%	100%	99%	66%	89%	90%	91%	98%	97%
4	100%	100%	100%	61%	92%	93%	56%	80%	82%	77%	92%	93%
3	98%	98%	97%	39%	87%	85%	55%	60%	61%	54%	88%	87%

Note: the germination rate was the average value of three times measurements.

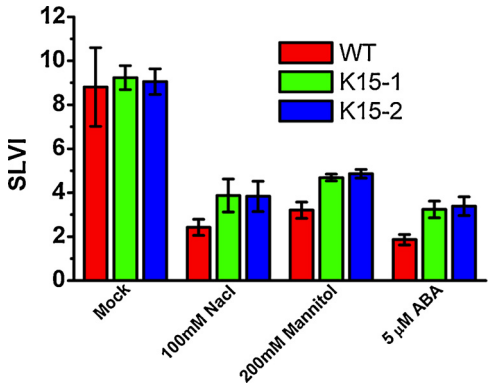

Fig. 4. Comparison of the seedling length vigor index (SLVI) between K15 and WT. **: $P \leq 0.01$; *: $P \leq 0.05$.

20% PEG6000 treatment. The MDA content in K15 leaves was significantly lower than that in WT leaves (Fig. 6C).

3.5. Multi-gene transformation affects the genes expression involved in stress Signal transduction

To investigate the effects of the transgenes on the gene expression of *B. napus* and further explain the mechanism of stress resistance in transgenic *B. napus*, we constructed the corresponding gene regulation networks based on the NCBI database (Fig. 7) for the five *Arabidopsis* genes that were transformed, namely, *AT3G26744*, *AT1G16540*, *AT4G25480*, *AT5G13630* and *AT3G14440*. The differentially expressed genes between K15 and WT plants under different stress conditions were detected by RNA sequencing (RNA-Seq). For all five target genes that were transferred, 33 genes had a regulatory relationship with them. Among these, *AT3G26744 (ICE1)* had a regulatory relationship with 20 genes, and this gene could regulate *AT4G33950 (OST1)*, which in turn had a regulatory relationship with *AT5G13630 (ABAR)*. *AT3G14440* (*NCED3*) was regulated by only one gene, *AT5G14760 (AO: L-aspartate oxidase);* for the *B. napus* homolog, there was no significant differential expression between K15 and WT lines. The transformed gene *AT3G26744 (ICE1)* regulates the gene *AT5G03240 (UBQ3),* whose homolog in *B. napus* is *BnaCnng72870D*. There was a significant differential expression of *BnaCnng72870D* between K15 and WT plants under the five treatment conditions. In K15 plants, *BnaCnng72870D* expression significantly increased under drought stress and significantly decreased under other treatments, with all the |log2| fold changes being higher than 3.4 times. On the other hand, in K15 plants, *novel_G001531* expression significantly decreased under all stress treatment except for drought stress. Compared with WT plants, expression of the homolog of *AT4G17730 (SYP23)* in *B. napus, BnaA01g08630D,* also decreased significantly in K15 plants under all stress treatment except for drought stress, with the |log2| fold changes being higher than 3.6 times (Supplementary Table S2 and S3).

For the five genes transformed in this investigation, 33 *Arabidopsis* genes had a regulatory relationship with them, 199 transcripts were found in our *B. napus* transcriptome data homologues to 33 *Arabidopsis* genes. We found that 78 of 199 transcripts were differentially expressed, and draw the heatmaps and perform cluster analysis based on log2(FC) values under the five treatments, as shown in Fig. 7B. It shows that the 78 DEGs can be classified into two categories. The first category tends to be down-regulated under the five treatments, while the other category tends to be up-regulated. Through differential expression analysis and pathway enrichment analysis, eight genes with significantly differential expression were screened out, and their distributions and expression level under all stress treatments are shown in Fig. 7C and Supplementary Table S2 and Supplementary Table S3. In particular, comparison of K15 and WT transcriptome in five stress treatments revealed two shared genes, *BnaCnng72870D* and *novel_G001531*, with a significant differential expression between WT and K15 plants in all five-stress treatments.

4. Discussion

In this study, multiple stress-responding genes, including *ABAR*, *NCED3*, *LOS5*, *CBF3* and *ICE1*, were cloned into a binary vector pABA-oriT and transformed *B. napus* to improve comprehensive stress resistance. Approximately 4,100 seeds were harvested. After selection and identification, 9 primary transformants were obtained for a transformation efficiency of 0.2%, which was much lower than the previous report for the single gene transformation [28]. The low efficiency might be due to two aspects: on the one hand, the multi-gene transformation vector is rather large in size, which increased the difficulty of genetic transformation. Consistence with this result, Chen et al reported that a large vector can readily lead to the ineffectiveness of heat shock transformation and electroporation transformation during the transformation of *Agrobacterium* strains [15]. On the other hand, given the nature of heterologous transformation in this work, the genetic difference and physiological status of the recipient materials are also important key factors that limit the efficiency of genetic transformation with the *Agrobacterium*-mediated flower-dip method. In Chen's experiment, this method was used to transfer the multi-gene transformation vector pMDC99-MAR-GUS-EGFP-MAR into *Arabidopsis* with a transformation efficiency of 0.3% [29]. Moreover, we have also attempted transformation in cotton using this pABA-oriT vector, so far we have not been able to obtain any transformants.

The results of the gene expression analysis showed various expression patterns among the five target genes in the transformants. The expression of *ICE1* and *LOS5,* which was driven by the *pSuper* promoter, was significantly (3 times) higher in the K15 transformants than that in WT plants under normal growth conditions (Fig. 2**B and D**). However, such expression levels were lower than those reported in *Arabidopsis* [15]. This difference might be due to the recipient materials were heterologous in the transformation in this work. A similar result was also reported by Lu [30] who used the *pSuper* promoter to drive the *Arabidopsis* gene *AtLOS5* to achieve heterologous expression in maize, and the fold increase of expression level was approximately 3.5 times. Moreover, the results of the gene expression analysis for the genes driven by the hypothetically constitutive *pSuper* promoter also showed that the expression of those genes in the K15 transformants could also be induced by stress, although less than that for genes driven by an

Table 2
The relative dry weight and relative fresh weight of seedlings.

Stress treatment	Relative dry weight average (%)			Relative fresh weight average (%)			*P* value by *t*-test	
	WT	K15-1	K15-2	WT	K15-1	K15-2	Relative dry weight	Relative fresh weight
100 mM NaCl	71.3	75.1	76.3	33.9	38.8	37.6	0.264	0.177
200 mM mannitol	55.5	57	58.1	28.5	32.8	33.5	0.608	0.027*
5 μM ABA	68.7	71	70.4	28.3	31.2	30.9	0.638	0.173

Note: * indicates significant differences between two samples by two-sample *t*-test, $P \leq 0.05$.

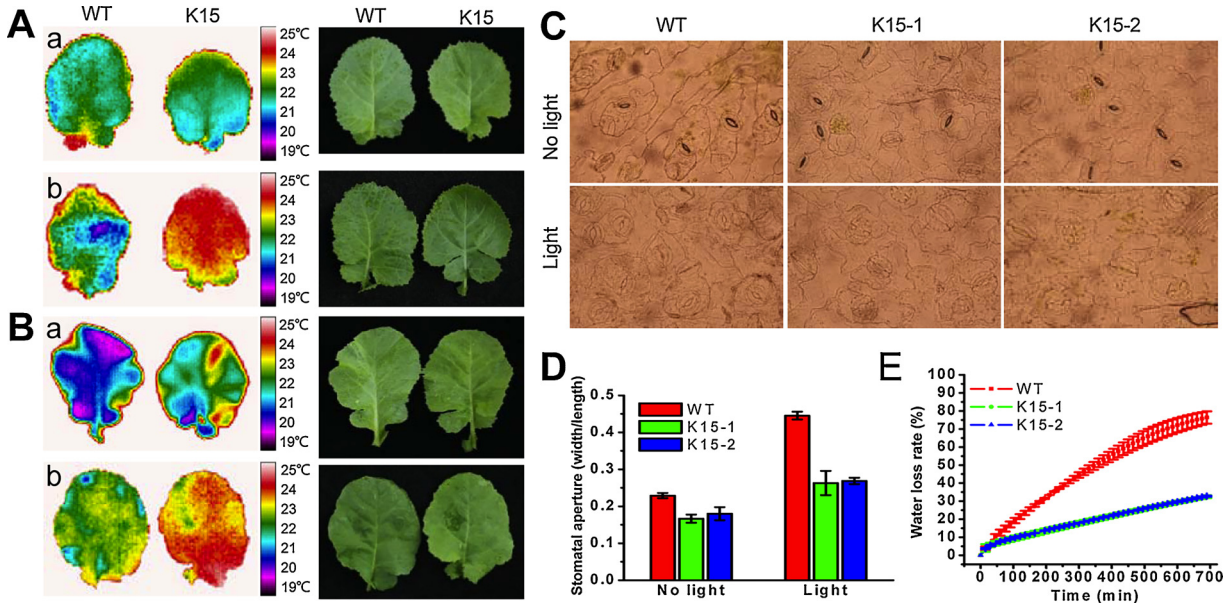

Fig. 5. Comparison of leaf temperature, stomatal aperture and water loss rate between K15 and WT. A: Leaf temperature for the second true leaf; B: Leaf temperature for the third true leaf. a. Soil water content of 35%; b. Soil water content of 5%; C: Stomatal aperture observation; D: Stomatal aperture measurement; E: water loss rate in leaves. **: $P \leq 0.01$; *: $P \leq 0.05$.

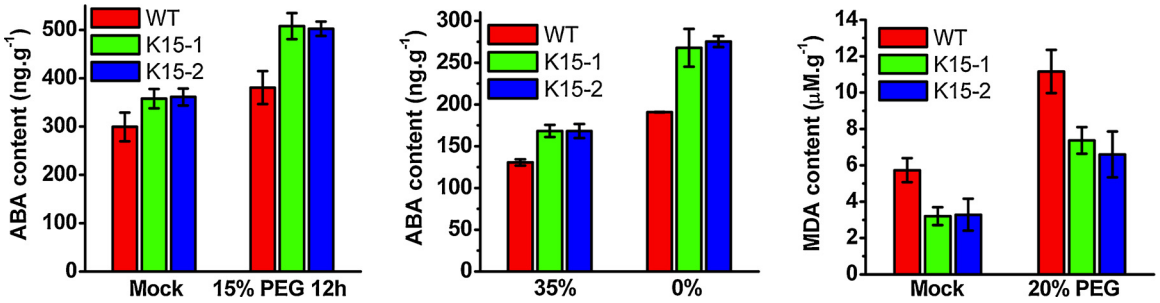

Fig. 6. Determination of ABA content and MDA content in WT and K15. A: The ABA content in the seedlings; B: The ABA content in the leaves; 35% and 0% are soil water content; C: The MDA content in the leaves. Mock: no stress treatment; **: $P \leq 0.01$; *: $P \leq 0.05$.

inducible promoter (Fig. 2**B and D**). Maybe the presence of induced expression caused by the inadequate rigorousness of constitutive promoter expression cannot be ruled out. Similar results have been previously reported by Chen et al [15]. In this work, it is noteworthy that the expression abundance of *ICE1* reached the peak at 12 h after stress treatment in K15 plants but at an earlier time in WT plants (Fig. 2**B and D**). This result may reflect the difference in expression patterns between the endogenous *ICE1* gene in *B. napus* and the transformed *ICE1* gene.

It is generally believed that *CBF3* and *ICE1* are the key genes involved in anti-freeze regulation [27]. In this work, the results of the gene expression analysis showed that *CBF3* and *ICE1* gene expression was also upregulated in WT plants under drought stress and salt stress (Fig. 2**B and D**). Previous studies have reported that by heterologous expression of *Arabidopsis CBF* transcription factors, the anti-freezing capacity can be enhanced and the tolerance to other stresses can be improved in transformants [25]. As early as in 2008, *ICE1* has been reported to control stomatal differentiation [31]. All these studies indicated that the functions of *CBF3* and *ICE1* are not limited to anti-freezing regulation. Although *ICE1* is generally thought to be constitutively expressed in cells and its expression level is co-regulated by post-translational ubiquitination and sumoylation [32], the aforementioned examples illustrated the diversity of expression patterns for *CBF3* and *ICE1* genes. In addition, *ICE1* can be modulated by phosphorylation of *OST1*, a key downstream kinase in the ABA regulatory pathway, to facilitate its binding with regulatory elements in the promoter region of *CBF3* [33]. That work can explain the results in the current study; that upregulated *CBF3* and *ICE1* gene expression in WT plants under drought stress and salt stress is likely due to the gene expression of *CBF3* and *ICE1* activated by the increase of ABA synthesis under adversity stress. While the specific activation pathway may be due to the interaction of OST1 and ICE1, other unknown pathways may exist, too.

Stress resistance analysis for plant growth in the germination and post-germination stages showed that under the conditions of 100 mM NaCl, 200 mM mannitol and 5 μM ABA, K15 exhibited significantly higher seed germination rates and sprouting percentages than the WT line. The K15 also showed pronounced post-germination growth potential compared with the WT (Table 1). This seems to contradict the current understanding of the role of ABA in the growth process during seed germination and post-germination stages, which generally holds that the ABA signaling pathway can activate the spatial- and temporal-specific expression of *ABI4* and *ABI5* in seeds at particular stages, thereby inhibiting growth after seed germination and post-germination. In this study, due to stress-induced expression or overexpression of *NCED3* and *LOS5*, K15 plants had significantly higher endogenous ABA content than WT plants (Fig. 6**A and B**), theoretically, it should inhibit seed germination and post-germination growth of K15. Such opposite results observed in K15 plants in this work (Table 1) are likely due to the simultaneous overexpression of the *ICE1* gene in K15. *ICE1* could negatively regulate the expression of *ABI4* and *ABI5*, two genes that are downstream of the ABA signaling pathway, thereby attenuating the

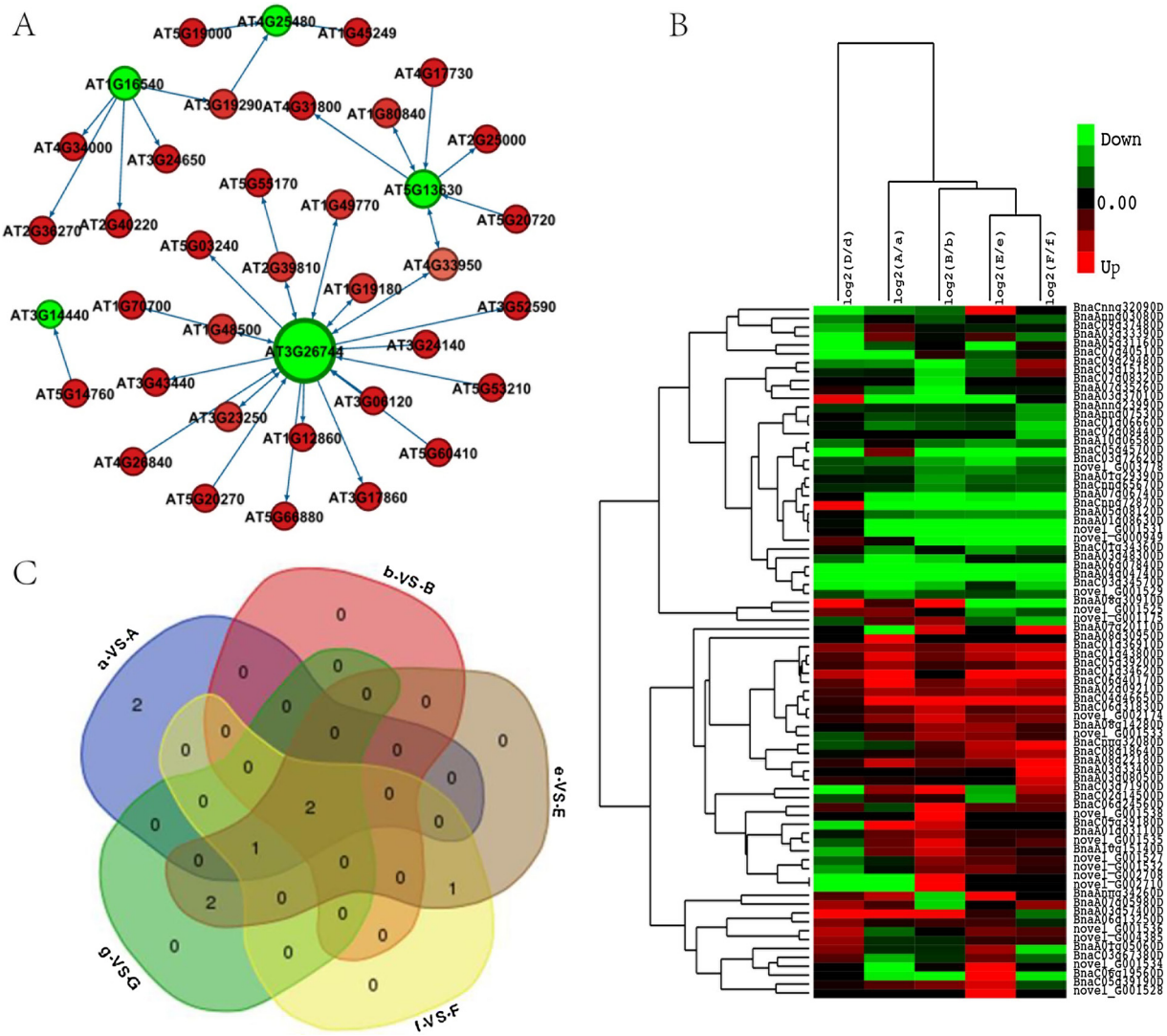

Fig. 7. The five target genes transformed in this study and their interactions in *Arabidopsis* (A), 78 DEGs heatmaps and cluster under the five treatments (B) and the Venn diagram for significantly differentially expressed genes under different stress conditions (C). The green nodes represent the five transformed genes, and the red nodes represent the 33 genes that interact with the five transformed genes (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

inhibitory effect of ABA on seed germination and on post-germination growth [34]. Hence, it is presumed that, due to the role of *ICE1* overexpression, the expression of the two negative control genes, *ABI4* and *ABI5,* was inhibited in process of seed germination and post-germination growth. Thus, plants would not exhibit the phenotypes caused by overexpression of ABA synthesis-related genes which can hypothetically inhibit seed germination and post-germination growth.

The ABA content in plants was determined by ELISA in this study. Even when there was no stress treatment, K15 plants already had a significantly higher ABA content in seedlings and leaves than WT plants (Fig. 6**A and B**). This can be attributed to the constitutive overexpression of the *LOS5* as an ABA synthesis-related gene in K15. After stress treatment, K15 plants had significantly higher ABA content than WT plants, which may have been due to the stress-induced overexpression of *NCED3*. Moreover, the results also showed that for both K15 and WT plants, the ABA content in the seedlings was significantly higher than that in the leaves (Fig. 6**A and B**). There are three possible reasons for this finding: i) the endogenous ABA content in different growth stages and in different plant tissues and organs might be different; ii) the residual ABA in the seeds might lead to higher ABA content in the seedlings than in the leaves; and iii) during seedling growth and/or stress treatment, hydroponic culture might affect gas exchange in the seedling roots and resulted in waterlogging, such that more ABA was accumulated in the seedlings. In addition, despite a significantly higher endogenous ABA content in K15 plants than in WT plants, the increased fold of ABA content was not proportional to the increased fold of target gene expression, but was vastly lower (Fig. 2**B and D**; Fig. 6**A and B**). Overexpression of *LOS5* driven by the *pSuper* promoter in maize resulted in ABA content increased by two times in the transformants with stress treatment [30], which was similar to the results in this study.

With high temperature and extreme drought treatment, even without low-temperature vernalization, K15 plants could still achieve a smooth transition from vegetative growth to reproductive growth and continued to complete their life cycle. In contrast, under the same condition, WT plants could not enter the reproductive growth stage (Fig. 3**A and B**). Recently, it has been reported that *ICE1* is also involved in the regulation of flowering by negatively regulating the expression of the *FLC* gene, and the activity of *ICE1* is regulated by the flowering regulator *SOC1*, too [35]. Therefore, it is not certain that such phenotypic differences observed in this study are due to the expression of a single gene or the combinatorial effect of multiple genes. It is

noteworthy that when plants grown in the greenhouse were subjected to continuous drought stress treatment, the phenotypic differences between K15 and WT plants were similar to those under high-temperature stress treatment (Fig. 3**A and B**). This indirectly suggests the presence of cross-talk in the regulation of plant stress resistance.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi: https://doi.org/10.1016/j.plantsci.2018.06.014.

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